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Comparison between effects of caffeine and ryanodine on electromechanical coupling in myocardium of hibernating chipmunks: role of internal Ca stores

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1 To clarify the cause of uncoupling of Ca influx through Ca channels and the contractility of the myocardium in hibernating chipmunks, the electromechanical effects of two different internal Ca store inhibitors, caffeine and ryanodine, and a cardiotonic agent, isoprenaline, were investigated in papillary muscles of hibernating animals.

2 Ryanodine (10^{-6} M), an inhibitor of internal Ca release, abolished the contraction with a marked inhibition of the action potential plateau (APp). In such preparations, an increase in Ca influx induced by isoprenaline (5×10^{-8} M) failed to augment the contraction, indicating uncoupling of Ca influx and contraction.

3 In ryanodine pretreated preparations, 10 mM caffeine produced an early phase of APp, but did not affect the contraction abolished by ryanodine, while a higher concentration of caffeine (25 mM) markedly increased the contraction with an augmentation of the electrical response.

4 In the absence of ryanodine, caffeine (5 mM) almost abolished the contraction with a greater inhibition of APp. In such preparations, isoprenaline greatly increased the contraction with an augmentation of the early phase of APp.

5 These effects were not significantly affected by additional application of ryanodine, but were inhibited by nifedipine, a Ca channel blocker.

6 These observations suggest that in cardiac muscles of hibernating animals, lack of the positive inotropic effect of isoprenaline may be attributed to a rapid and effective sequestration of increased cytoplasmic Ca through Ca influx by internal stores, probably by enhancement of their ability to take up Ca.

Introduction

From previous studies on the myocardium of hibernating animals (Charnock *et al.*, 1983; Kondo & Shibata, 1984; Kondo, 1986a,b; 1987a) it has been suggested that the relative contributions of different sources of Ca for activation of contraction (Ca influx and Ca release from internal stores) are changed during hibernation. In hibernating preparations, the Ca source is mainly attributed to the release from intracellular Ca stores. Recently, in cardiac muscles of hibernating chipmunks, it has been found that the positive inotropic effect of isoprenaline was not observed in spite of a marked augmentation of Ca influx through Ca channels (Kondo, 1986b). To explain this uncoupling of Ca influx and the contractile force, an increase in the ability of internal Ca stores, sarcoplasmic reticulum (SR), to sequester cytoplasmic Ca has been suggested (Kondo, 1986b). This assumed mechanism may effectively sequester cytoplasmic Ca increased through Ca influx across cell membranes, resulting in a lack of the positive inotropic effect of isoprenaline. If this is so, inhibition of Ca uptake by the SR may restore the activation of contraction coupled with Ca influx.

There are two inhibitors of function of SR, ryanodine and caffeine, which are useful for studying the excitation-contraction coupling mechanisms. Ryanodine is thought to exert its effects through inhibition of the Ca releasing process from SR (Sutko & Kenyon, 1983; Chamberlain *et al.*, 1984; Marban & Wier, 1985; Fabiato, 1985), while it has been suggested that the inhibitory action of caffeine is due to an inhibition of Ca uptake by SR (Weber & Herz, 1968; Blinks *et al.*, 1972; Henderson *et al.*, 1974; Hess & Wier, 1984). Caffeine has also been reported to augment Ca influx through Ca channels (Schneider & Sperelakis, 1975; Goto *et al.*, 1979).

In the present experiments with these drugs, I examined the involvement of SR function in cardiac excitation-contraction coupling of hibernating animals. The present findings are consistent with my previous hypothesis that the ability of the SR to take up calcium is enhanced in hibernating preparations.

Methods

Asian chipmunks (*Tamias sibiricus*) of either sex were trapped in September and transferred to individual wiremesh cages. They were introduced to a darkened cold room (4 ± 1°C) with food, a standard diet of pelleted laboratory rat chow, and water available. Most of them had exhibited preliminary bouts of hibernation within 3 weeks, and subsequently they exhibited several consecutive bouts of hibernation longer than 1 week in duration until the following March. Animals in deep hibernation were used for experiments.

Animals were killed following cervical dislocation. The heart was quickly excised, a papillary muscle, 2–3 mm in length and less than 1 mm in diameter, was isolated from the right ventricle. The preparation was mounted and equilibrated for 2 h in a tissue bath containing Krebs-Ringer solution aerated with 95% O_2 and 5% CO_2, with the ends of the muscle impaled on two hooks, one of which was attached to a force displacement transducer (Kondo, 1987a). The composition of the Krebs-Ringer solution (mM) was: NaCl 120, KCl 4.8, $CaCl_2$ 1.2, $MgSO_4 \cdot 7H_2O$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 24.2 and glucose 5.5 (pH 7.4). The temperature of the superfusate was maintained at 30°C. The preparations were stimulated at 0.2 Hz with pulses 1 ms in duration and twice the diastolic threshold. Membrane action potentials were recorded through glass microelectrodes filled with 3 M KCl. The action potential and the mechanical tension were simultaneously displayed on a storage oscilloscope (Tektronix 7613). The mechanical tension was also recorded on a polygraph (Nihon Kohden TB612T).

The agents employed in the experiments were as follows: isoprenaline (5×10^{-8} M; Sigma, St Louis, MO, U.S.A.), caffeine (5–25 mM; Wako P.C.I. Ltd, Osaka, Japan), ryanodine (10^{-6} M; S.B. Penick Co, New York, U.S.A.) and nifedipine (10^{-6} M; Bayer).

Results

In papillary muscles of hibernating animals, ryanodine (10^{-6} M) markedly inhibited the action potential plateau (APp) and abolished the contraction (Figure 1). In such preparations, additional application of isoprenaline (5×10^{-8} M) greatly augmented APp, but did not affect the contraction. In the present preparations, uncoupling of Ca influx through the action potential depolarization and the contraction was observed. This uncoupling of the electrical and the mechanical responses has been found in my previous study (Kondo, 1986b).

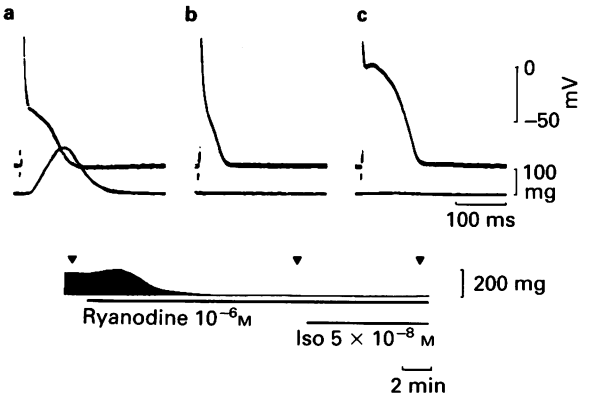

Figure 1 Electromechanical effects of isoprenaline in the presence of ryanodine. Top trace and middle trace show the simultaneous recordings of the action potential and the contraction. The bottom trace shows the continuous recording of the developed tension. (a) Control; (b) 15 min after application of ryanodine (10^{-6} M); (c) additional application of isoprenaline (5×10^{-8} M) for 9 min. Arrowheads indicate recording points of the electrical response.

In the presence of ryanodine, the effects of caffeine were examined in four preparations (Figure 2). After inhibition of APp and the contraction by ryanodine, caffeine (10 mM) caused an increase in APp at an early phase but had little effect on the contraction which had been abolished by ryanodine. The additional application of a higher concentration of caffeine (25 mM) further increased the APp and simultaneously activated the contraction. The activation of contraction was characterized by a marked

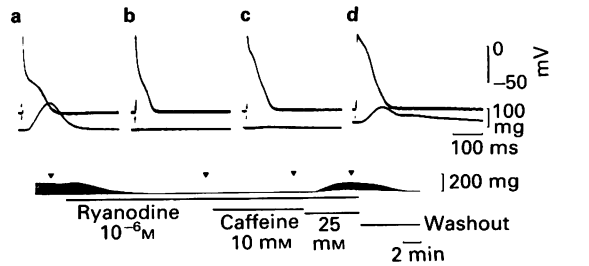

Figure 2 Electromechanical effects of caffeine in the presence of ryanodine. (a) Control; (b) 15 min after application of ryanodine (10^{-6} M); (c and d) additional application of caffeine (c, 10 mM for 9 min and d, 25 mM for 5 min).

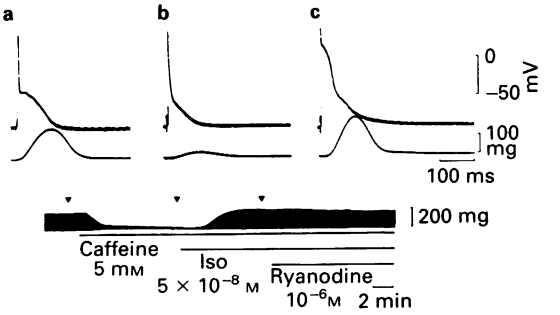

Figure 3 Electromechanical effects of caffeine and ryanodine on the response to isoprenaline. (a) Control; (b) 10 min after application of caffeine (5 mM); (c) additional application of isoprenaline (5×10^{-8} M). After treatment with caffeine and isoprenaline, ryanodine (10^{-6} M) was applied for 12 min.

prolongation of the active state due to a decrease in the rate of relaxation. Under these conditions, the resting tension also rose. After caffeine was washed out, the effects of this drug were rapidly removed.

The effects of caffeine on the responses to isoprenaline in the absence of ryanodine were examined in four preparations (Figure 3). A lower concentration of caffeine (5 mM) caused a marked inhibition of contraction with a decrease in APp. Such inhibitory effects on the electrical and the mechanical responses were similar to those of ryanodine. Caffeine slightly affected APp at an early phase. In this preparation, isoprenaline caused a marked positive inotropic effect accompanied by an augmentation of APp at an early phase. The positive inotropic effect of isoprenaline in the presence of caffeine was not substantially affected by additional application of ryanodine. Nifedipine (10^{-6} M), a Ca channel blocker, inhibited the electrical and the mechanical response to isoprenaline (not shown).

Discussion

In cardiac muscles of hibernating chipmunks, the following characteristics have been found: (1) the contraction and the action potential plateau (APp) are controlled by internal Ca release from sarcoplasmic reticulum (SR) (Kondo & Shibata, 1984; Kondo, 1986b); (2) the activation of Ca channels is inhibited by development of a large transient outward current activated by membrane depolarization, resulting in an abolition of the early plateau phase of the action potential (Kondo, 1986a; 1987a). From these results, it has been suggested that the cardiac electromechanical responses of hibernating animals depend on internal Ca release. In these preparations, isoprenaline, a β-adrenoceptor agonist, has been shown to augment Ca influx through facilitation of Ca channel activation, but not to increase the contractile force (Kondo, 1986b). A lack of effect of isoprenaline on contraction has also been reported in rat cardiac cells, the contraction of which is thought to depend largely on Ca release from SR (Fabiato, 1981). However, in myocardium of non-hibernating animals and other mammals, this electromechanical uncoupling has not been observed, suggesting the existence of an internal system strongly buffering cytoplasmic Ca in hibernating preparations. In a previous study (Kondo, 1986b), it was suggested that this uncoupling results from an enhancement of the ability of the SR to sequester Ca. Therefore, in order to test this hypothesis, such an uncoupling of Ca influx and the contractile force was examined in the presence of two different SR inhibitors, ryanodine and caffeine.

Ryanodine and caffeine may exert their negative inotropic effect on cardiac muscles by decreasing Ca release from SR. Several studies on the isolated cardiac SR vesicles (Blayney *et al.*, 1978), skinned cardiac preparations (Fabiato & Fabiato, 1973), cultured heart cells (Rasmussen *et al.*, 1987) and intact myocardium (Weber & Herz, 1968; Blinks *et al.*, 1972; Henderson *et al.*, 1974; Hess & Wier, 1984) have indicated that this decrease in Ca release by caffeine may be attributed to inhibition of Ca uptake by SR which results in the depletion of SR Ca. In contrast to caffeine, ryanodine has been reported not to inhibit Ca uptake by cardiac SR (Lattanzio *et al.*, 1987; Jones *et al.*, 1979; 1981). High concentrations of ryanodine directly inhibit Ca-induced Ca release from cardiac SR (Chamberlain, 1984) and in skinned cardiac cells (Fabiato, 1985). Other recent studies in isolated cardiac SR vesicles (Meissner, 1986; Lattanzio *et al.*, 1987) and single SR release channels (Rousseau *et al.*, 1987) have shown that submicromolar ryanodine increased Ca permeability of cardiac SR membrane probably by forming a subconductance state of the Ca release channel, resulting in depletion of releasable Ca from SR. This may account for a decrease in Ca release from SR by ryanodine. Thus, the two inhibitors may reduce cardiac SR Ca release through different processes.

In hibernating preparations, when the slow Ca inward current is activated by the Ca channel activator, isoprenaline, membrane depolarization generated by this current is clearly observed in the range above the threshold potential for the slow inward current (near −40 mV: the early plateau phase) (Kondo, 1986a,b). This potential change is more clearly observed in the presence of ryanodine (Kondo, 1986a,b), since some currents activated by internal Ca release (transient inward and outward currents, and Na–Ca exchange current) which normally affect the plateau potential are without effect

due to the reduced contribution of these currents because of the inhibition of internal Ca release by ryanodine. Furthermore, in the presence of ryanodine, a contraction activated by Ca influx is selectively observed. In such ryanodine-pretreated preparations, caffeine induced the early plateau phase of the action potential probably due to an increase in the slow Ca inward current (Shine & Langer, 1972; Schneider & Sperelakis, 1975; Goto *et al.*, 1979), indicating an increase in Ca influx. Under these conditions, caffeine (10 mM) did not significantly increase the contraction in much the same way as observed in the preparations treated with isoprenaline. However, a higher concentration of caffeine (25 mM) caused a marked increase in the contraction accompanied by a decrease in the rate of relaxation and a rise of the resting tension. These findings may be explained by a balance between the dual action of caffeine, an augmentation of Ca influx (Shine & Langer, 1972; Schneider & Sperelakis, 1975; Goto *et al.*, 1979) and an inhibition of SR Ca uptake (Blinks *et al.*, 1972; Henderson *et al.*, 1974; Hess & Wier, 1984; Weber & Herz, 1986; Rasmussen *et al.*, 1987). Since the effect of a higher concentration of caffeine on Ca influx and SR Ca uptake may be more pronounced, a part of Ca entering the cell under the influence of caffeine cannot be trapped in SR because Ca uptake is reduced. Untrapped Ca accumulates around the contractile proteins and activates the contraction, resulting in the contraction coupled with Ca influx. Subsequently, the diminished reuptake of Ca by SR causes a decrease in the rate of relaxation and, ultimately, the resting tension is raised (Shine & Langer, 1972). These theories are in good agreement with the effects of caffeine in the present experiments. These results suggest that a large capacity for Ca uptake of the SR is responsible for the uncoupling of Ca entry via Ca channels and the contractile force in hibernating preparations.

The above interpretation was strongly supported by the finding that the positive inotropic effect of isoprenaline was dramatically restored in the presence of caffeine. In the absence of ryanodine, a lower concentration of caffeine (5 mM) nearly abolished the contraction and APp without significantly affecting the early plateau phase of the action potential, suggesting that in the present preparations, this concentration of caffeine mainly exerts an inhibitory effect on SR. These electromechanical responses to caffeine are similar to those of ryanodine. However, under these conditions, the effect of isoprenaline on the mechanical response was markedly different from that in the presence of ryanodine. Isoprenaline caused a marked positive inotropic effect accompanied by an increase in the early plateau phase of the action potential. This effect was sensitive to nifedipine (a Ca channel blocker) but insensitive to ryanodine, suggesting that the increased mechanical response is associated with Ca influx through Ca channels. These indicate that in the presence of caffeine SR could not sequester the augmented cytoplasmic Ca induced by isoprenaline, because of an inhibition of SR Ca uptake.

In the present experiments, the involvement of an increasing effect of caffeine on the Ca sensitivity of the contractile proteins (Fabiato & Fabiato, 1976; Endo & Kitazawa, 1978; Wendt & Stephenson, 1983; Hess & Wier, 1984; Eisner & Valdeolmillos, 1985) may be unimportant. In the presence of ryanodine, caffeine 10 mM did not affect the contraction although it augmented Ca influx during the early plateau depolarization. A lower concentration of caffeine (5 mM) almost abolished the contraction and APp, both of which are controlled by Ca release from SR. Furthermore, it has been reported that caffeine increased the tonic component of tension following the twitch through an increase in Ca sensitivity of the contractile system while decreasing the twitch through an inhibition of SR function (Eisner & Valdeolmillos, 1985). The present caffeine effect was observed on the twitch. These observations suggest that the major mode of action of caffeine in the present experiments is not an increase in Ca sensitivity, although it is possible that this factor may contribute to some extent.

The present evidence indicates that caffeine removes an uncoupling of Ca influx through Ca channels and the contractile force in cardiac muscles of hibernating animals, by reducing Ca sequestration in the SR. It seems reasonable to conclude that this characteristic is markedly different from that observed in nonhibernating chipmunks and other mammals and results from an increased ability of the SR to take up Ca. Such a change in SR function may be closely associated with hibernation. Furthermore, in a previous study (Kondo, 1987b), the changes in cardiac function of chipmunks appeared to have been triggered before hibernation began, suggesting the possible involvement of some responsible substance(s). It is assumed that such substance(s) may also be involved in the regulation of the Ca uptake activity of the SR. Chipmunks are an important model for studying the function of SR in cardiac muscles.

References

BLAYNEY, L.B., THOMAS, H., MUIR, J. & HENDERSON, A. (1978). Action of caffeine on calcium transport by isolated fractions of myofibrils, mitochrondria, and sarcoplasmic reticulum from rabbit heart. *Circ. Res.*, **43**, 520–526.

BLINKS, J.R., OLSON, C.B., JEWELL, B.R. & BRAVENY, P. (1972). Influences of caffeine and other methylxanthines on mechanical properties of isolated mammalian heart muscle. *Circ. Res.*, **30**, 367–392.

CHAMBERLAIN, B.K., VOLPE, V. & FLEISCHER, S. (1984). Inhibition of calcium-induced calcium release from purified cardiac sarcoplasmic reticulum vesicles. *J. Biol. Chem.*, **259**, 7547–7553.

CHARNOCK, J.S., DRYDEN, W.F. & MARSHALL, R.J. (1983). Seasonal variations in drug response and staircase phenomena in atrial muscle from a hibernating rodent (*Spermophilus richardsonii*). *Br. J. Pharmacol.*, **78**, 151–158.

EISNER, D.A. & VALDEOLMILLOS, M. (1985). The mechanism of the increase of tonic tension produced by caffeine in sheep cardiac purkinje fibers. *J. Physiol.*, **364**, 313–326.

ENDO, M. & KITAZAWA, T. (1978). Excitation-contraction coupling in chemically skinned fibers of cardiac muscle. In *Proceedings of the VIII World Congress of Cardiology*. ed. Hayase, S. & Murao, S. pp. 800–803. Amsterdam: Excerpta Medica.

FABIATO, A. (1981). Myoplasmic free calcium concentration reached during the twitch of an intact isolated cardiac cell and during calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned cardiac cell from the adult rat or rabbit ventricle. *J. Gen. Physiol.*, **78**, 457–497.

FABIATO, A. (1985). Effects of ryanodine in skinned cardiac cells. *Fed. Proc.*, **44**, 2970–2976.

FABIATO, A. & FABIATO, F. (1973). Activation of skinned cardiac cells. Subcellular effects of cardioactive drugs. *Eur. J. Cardiol.*, **1**, 143–155.

FABIATO, A. & FABIATO, F. (1976). Techniques of skinned cardiac cells and of isolated cardiac fibres with disrupted sarcolemmas with reference to the effects of catecholamines and of caffeine. *Recent Adv. Stud. Card. Struct. Metab.*, **9**, 71–94.

GOTO, M., YATANI, A. & EHARA, T. (1979). Interaction between caffeine and adenosine on the membrane current and tension component in bullfrog atrial muscle. *Jap. J. Physiol.*, **29**, 393–409.

HENDERSON, A.H., BRUTSAERT, D.L., FORMAN, R. & SONNENBLICK, E.H. (1974). Influence of caffeine on force development and force frequency relations in cat and rat heart muscle. *Cardiovasc. Res.*, **8**, 162–172.

HESS, P. & WIER, W.G. (1984). Excitation-contraction coupling in cardiac purkinje fibers. Effects of caffeine on the intracellular $[Ca^{2+}]$ transient, membrane currents and contraction. *J. Gen. Physiol.*, **83**, 417–433.

JONES, L.R., BESCH, Jr. H.R., SUTKO, J.L. & WILLERSON, J.T. (1979). Ryanodine-induced stimulation of net Ca uptake by cardiac sarcoplasmic reticulum vesicles. *J. Pharmacol. Exp. Ther.*, **209**, 48–55.

JONES, L.R. & CALA, S.E. (1981). Biochemical evidence for functional heterogeneity of cardiac sarcoplasmic reticulum vesicles. *J. Biol. Chem.*, **256**, 11809–11818.

KONDO, N. (1986a). Excitation-contraction coupling in hibernating chipmunks myocardium. *Experientia*, **42**, 1220–1222.

KONDO, N. (1986b). Excitation-contraction coupling in myocardium of nonhibernating and hibernating chipmunks: Effects of isoprenaline, a high calcium medium and ryanodine. *Circ. Res.*, **59**, 221–228.

KONDO, N. (1987a). Electrophysiological effects of Ca antagonists, tetrodotoxin, $[Ca]_0$ and $[Na]_0$ on myocardium of hibernating chipmunks: possible involvement of Na-Ca exchange mechanism. *Br. J. Pharmacol.*, **91**, 315–319.

KONDO, N. (1987b). Identification of pre-hibernating state in myocardium from nonhibernating chipmunks. *Experientia*, **43**, 873–875.

KONDO, N. & SHIBATA, S. (1984). Calcium source for excitation-contraction coupling in myocardium of nonhibernating and hibernating chipmunks. *Science*, **225**, 641–643.

LATTANZIO, Jr. F.A., SCHLATTERER, R.G., NICAR, M., CAMPBELL, K.P. & SUTKO, J.L. (1987). The effects of ryanodine on passive calcium flux across sarcoplasmic reticulum membranes. *J. Biol. Chem.*, **262**, 2711–2718.

MARBAN, E. & WIER, W.G. (1985). Ryanodine as a tool to determine the contribution of calcium entry and calcium release to the calcium transient and contraction of cardiac purkinje fibers. *Circ. Res.*, **56**, 133–138.

MEISSNER, G. (1986). Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *J. Biol. Chem.*, **261**, 6300–6306.

RASMUSSEN, Jr. C.A.F., SUTKO, J.L. & BARY, W.H. (1987). Effects of ryanodine and caffeine on contractility, membrane voltage, and calcium exchange in cultured heart cells. *Circ. Res.*, **60**, 495–504.

ROUSSEAU, E., SMITH, J.S. & MEISSNER, G. (1987). Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channel. *Am. J. Physiol.*, **253**, C364–C368.

SCHNEIDER, J.A. & SPERELAKIS, N. (1975). Slow Ca^{2+} and Na^{2+} responses induced by isoproterenol and methylxanthines in isolated perfused guinea pig hearts exposed to elevated K^+. *J. Mol. Cell. Cardiol.*, **7**, 249–273.

SHINE, K.I. & LANGER, G.A. (1972). Caffeine effects upon contraction and calcium exchange in rabbit myocardium. *J. Mol. Cell. Cardiol.*, **3**, 255–270.

SUTKO, J.L. & KENYON, J.L. (1983). Ryanodine modification of cardiac muscle responses to potassium-free solutions. Evidence of inhibition of sarcoplasmic reticulum calcium release. *J. Gen. Physiol.*, **82**, 385–404.

WEBER, A. & HERZ, R. (1968). The relationship between caffeine contracture in intact muscle and the effect of caffeine on reticulum. *J. Gen. Physiol.*, **52**, 750–759.

WENDT, I.R. & STEPHENSON, D.G. (1983). Effects of caffeine on Ca-activated force production in skinned cardiac and skeletal fibers of the rat. *Pflügers Arch.*, **398**, 210–216.

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